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MOLECULAR AND MORPHOMETRIC EVIDENCE FOR SEPARATE SPECIES OF *UNCINARIA* (NEMATODA: ANCYLOSTOMATIDAE) IN CALIFORNIA SEA LIONS AND NORTHERN FUR SEALS: HYPOTHESIS TESTING SUPPLANTS VERIFICATION

Steven A. Nadler, Byron J. Adams*, Eugene T. Lyons†, Robert L. DeLong‡, and Sharon R. Melin‡
Department of Nematology, University of California, Davis, California 95616-8668

ABSTRACT: California sea lions (*Zalophus californianus*) and northern fur seals (*Callorhinus ursinus*) are each believed to host distinct hookworm species (*Uncinaria* spp.). However, a recent morphometric analysis suggested that a single species parasitizes multiple pinniped hosts, and that the observed differences are host-induced. To explore the systematics of these hookworms and test these competing hypotheses, we obtained nucleotide sequences of nuclear ribosomal DNA (D2/D3 28S, D18/D19 28S, and internal transcribed spacer [ITS] regions) from 20 individual hookworms parasitizing California sea lion and northern fur seal pups where their breeding grounds are sympatric. Five individuals from an allopatric population of California sea lions were also sampled for ITS-1 and D18/D19 28S sequences. The 28S D2/D3 sequences showed no diagnostic differences among hookworms sampled from individual sea lions and fur seals, whereas the 28S D18/D19 sequences had one derived (apomorphic) character demarcating hookworms from northern fur seals. ITS sequences were variable for 7 characters, with 4 derived (apomorphic) states in ITS-1 demarcating hookworms from California sea lions. Multivariate analysis of morphometric data also revealed significant differences between nematodes representing these 2 host-associated lineages. These results indicate that these hookworms represent 2 species that are not distributed indiscriminately between these host species, but instead exhibit host fidelity, evolving independently with each respective host species. This evolutionary approach to analyzing sequence data for species delimitation is contrasted with similarity-based methods that have been applied to numerous diagnostic studies of nematode parasites.

Stiles and Hassall (1899) originally reported hookworms (*Uncinaria*) of northern fur seals, *Callorhinus ursinus* Linneaus, from the Pribilof Islands in the Bering Sea. These specimens were later named *Uncinaria lucasi* Stiles (Stiles, 1901). However, because the description was unsatisfactory and the type material damaged, Baylis (1947) redescribed *U. lucasi* using new specimens obtained from *C. ursinus* (syn. *C. alascanus*, *Callotaria alascana*), also collected from the Pribilof Islands (St. Paul Island). *Uncinaria lucasi* has been reported primarily from species of fur seals, although exceptions include a report from Steller's sea lion, *Eumetopias jubatus* Schreber (Olsen, 1958), and a South American sea lion *Otaria byronia* Blainville (George-Nascimento et al., 1992).

Uncinaria lucasi is the only pinniped hookworm for which detailed life-cycle information is known (Olsen 1958, 1974; Olsen and Lyons, 1965; Lyons and Keyes, 1978, 1984; Lyons and Bigg, 1983; Lyons et al., 1997). Unlike hookworms from most terrestrial hosts, infections of adult *U. lucasi* occur only from parasitic L_3 larvae passed to nursing pups in their mother's first milk. Adult hookworms are eliminated spontaneously from pups approximately 3 mo postinfection. Hookworm eggs develop to free-living L_3 larvae in rookery soil, and L_3 penetrate the skin of seals, or enter orally, and persist in tissues. The life cycle is completed when parasitic L_3 are reactivated within lactating fur seals and transmitted to pups via milk. Hookworms in California sea lions are assumed to have a similar life cycle, and although no experimental infections have been completed, circumstantial evidence supports this hypothesis (Lyons and Keyes, 1984; Lyons et al., in press).

The other species of hookworm described from otariids (fur seals and sea lions) is *U. hamiltoni* Baylis, obtained from *O. byronia* at Cape Dolphin in the Falkland Islands (Baylis, 1947). Baylis (1933) originally suggested that the specimens of *Uncinaria* from *O. byronia* were conspecific with specimens recovered from what he later asserted (Baylis, 1947) was a California sea lion *Zalophus californianus* Lesson (in 1933, he indicated uncertainty regarding whether the host was a California sea lion or a northern sea lion *E. jubatus*, syn. *E. stelleri*). Baylis (1947) and Dailey and Hill (1970) stated that specimens from *Z. californianus* had characteristics intermediate between *U. lucasi* and *U. hamiltoni. Uncinaria hamiltoni* has also been reported from the Australian sea lion *Neophoca cinerea* Péron and Lesueur (Beveridge, 1980). These descriptions and reports of *Uncinaria* have led to ambiguity concerning the presence of distinct hookworm species among different species of otariids.

A comparison of differences between *U. lucasi* and *U. hamiltoni*, according to their descriptions (Baylis, 1933, 1947), depicts *U. lucasi* as having: (1) a smaller buccal capsule with a slightly different shape than *U. hamiltoni*; (2) no thickening of the buccal capsule wall at its base, and no toothlike structures at the subdorsal angles of the border of the capsule; (3) smaller subventral teeth; (4) a shorter esophagus; and (5) a smaller bursa. *U. hamiltoni* has also been reported to have a shorter anterolateral ray relative to the other 2 rays, and the spicules of this species are described as being twice as long as those of *U. lucasi*. Other differences, related to hosts, have been reported more recently. For example, L_3 hatched from eggs of hookworms from California sea lion pups are approximately 100 μm shorter than those from northern fur seal pups (Lyons and Keyes, 1978), adults are distributed differently in the intestinal tracts of these hosts (Lyons and Keyes, 1984; Lyons et al., 1997), California sea lion pups exhibit intestinal petechiae that are more diffuse (Lyons et al., 1997), and adult hookworms live at least twice as long in California sea lions (Lyons et al., in

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* Department of Entomology and Nematology, University of Florida, Gainesville, Florida 32611-0620.
† Department of Veterinary Science, Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky 40546-0099.
‡ National Marine Mammal Laboratory, Alaska Fisheries Science Center, National Marine Fisheries Service, NOAA, Sand Point Way, N.E., Seattle, Washington 98115-0070.

press). George-Nascimento et al. (1992) found differences in nematode body size, prevalence of host skin lesions, and intensity of infection for hookworms parasitizing South American sea lions (*O. byronia*) and South American fur seals (*Arctocephalus australis* Zimmermann). They concluded that these differences were all host-induced and opined that there is ''just one widely distributed hookworm species (*Uncinaria lucasi* Stiles . . .), with different host races and geographic subspecies.'' (George-Nascimento et al., 1992).

In the present study, we investigated the specific status of *Uncinaria* taxa parasitizing 2 otariid species, the California sea lion and the northern fur seal. Morphometric features of these hookworm taxa were compared using multivariate statistical methods. Individual hookworms, collected primarily from rookeries where the host species are sympatric, were sequenced for regions of nuclear ribosomal DNA (2 regions of the 28S subunit, and internal transcribed spacers [ITS]) and the distribution of character states among individuals used to assess lineage exclusivity and species status. The results and logic of this approach are contrasted to similarity-based methods that have been used to investigate nematode species using sequence data (e.g., Hung et al., 1997, 1999; Newton et al., 1998; Gasser et al., 1999; Heise et al., 1999).

MATERIALS AND METHODS

Uncinaria specimens were collected from hosts (otariid pups) on San Miguel and San Nicolas Islands, California, as described in Lyons et al. (1997). *Uncinaria stenocephala* Railliet was collected from an Arctic fox (*Alopex lagopus* Linneaus) from St. Paul Island, Alaska. *Necator americanus* Stiles were obtained from human hosts in Guatemala following anthelmintic treatment. Specimens collected for molecular analysis were preserved in 95–100% ethanol at field collection sites and stored in ethanol at −20 C when returned to the laboratory. Specimens collected for morphological analysis were fixed and stored in alcohol–formalin–glycerine. Vouchers of the specimens used for morphological (UCDNC 3666–3668) and molecular (anterior and posterior ends only; voucher numbers in Table I) study were deposited in the University of California Davis Nematode Collection.

Measurements were made for 25 male and 25 female hookworms collected from each of 3 pups (1 northern fur seal pup from St. Paul Island, Alaska collected on 10 August 1960, 1 northern fur seal pup from San Miguel Island, California collected on 21 July 1996, and 1 California sea lion pup from San Miguel Island, California collected on 18 July 1996). Measurements for males included total length, width, buccal capsule depth, esophageal length, and spicule length (Table II); the first 4 of these measurements were also made for females, as was distance of vulva from the posterior end, and tail length (Table III). MANOVA, using Wilkes Lambda statistics, was used for comparison of these measurements (by sex of nematode) from each of the 3 hosts from the 2 localities.

DNA was extracted from the excised midbody of 25 individual hookworms (16 females, 9 males) obtained from 7 California sea lion pups and 6 northern fur seal pups (Table I) using a DNA binding (''glass milk'') method involving isothiocyanate and guanidinium (ID Pure Genomic DNA Kit, ID Labs Biotechnology; London, Ontario, Canada). Twenty of these hookworms were obtained from sea lion or fur seal pups raised at sites where these 2 host species are sympatric (share the same rookery areas and soil, and thus are likely to be exposed to common L_3); 5 hookworms (4 females and 1 male) were obtained from 2 California sea lion hosts on San Nicolas Island where fur seals do not co-occur (Table I). A region of nuclear ribosomal DNA (rDNA), including the 18S 3′-end, ITS-1 and ITS-2, 5.8S subunit, and 28S 5′-end was amplified using the polymerase chain reaction (PCR). The strategy for design of ITS PCR primers (sequence positions according to *Caenorhabditis elegans* numbering, Ellis et al, 1986; GenBank X03680) was described previously (Nadler et al., 2000). These primers anneal to the 3′-end of the 18S rDNA (forward [>] primer no. 93, 2,635–2,653 5′-TTGAACCGGGTAAAAGTCG) and 5′-end of the 28S rDNA (reverse [<] primer no. 94, 3,745–3,764 5′-TTAGTTTCTTTTCCTCCG CT). DNA from the D2/D3 region of the 28S nuclear ribosomal subunit was amplified by PCR using > primer no. 500 (4,078–4,100 5′-AC TTTGAAGAGAGAGTTCAAGAG) and < primer no. 501 (4,681–4,700 5′-TCGGAAGGAACCAGCTACTA). DNA from the D18/D19 region of the 28S nuclear ribosomal subunit was amplified by PCR using > primer no. 527 (5,003–5,026 5′-CTAAGGAGTGTGTAAC AACTCACC) and < primer no. 532 (6,067–6,090 5′-AATGACGA GGCATTTGGCTACCTT). Cycling parameters for the ITS rDNA amplifications consisted of an initial denaturation at 94 C for 3 min, followed by 33 cycles of 94 C for 30 sec, 56 C for 30 sec, and 72 C for 75 sec, and a postamplification extension at 72 C for 5 min. Cycling parameters for the D2/D3 rDNA amplifications consisted of an initial nucleic acid denaturation at 94 C for 3 min, followed by 33 cycles of 94 C for 30 sec, 54 C for 30 sec, and 72 C for 1 min, and a postamplification extension at 72 C for 7 min. Cycling parameters for the D18/D19 28S rDNA amplifications consisted of an initial nucleic acid denaturation at 94 C for 3 min, followed by 34 cycles of 94 C for 30 sec, 54 C for 30 sec, and 72 C for 45 sec, and a postamplification extension at 72 C for 7 min. PCR amplifications were performed using a proofreading DNA polymerase (ID Proof, ID Labs Biotechnology, or Finnzymes DyNAzyme EXT, MJ Research, Watertown Massachusetts), 0.5 μM of each primer, 200 μM deoxynucleoside triphosphates, and a $MgCl_2$ concentration of 2 mM (D2/D3 reactions) or 3 mM (D18/D19 28S and ITS reactions) in a total reaction volume of 25 μl. Templates for direct sequencing of amplified DNA from individual nematodes were prepared by enzymatic treatment of PCR products using exonuclease I and shrimp alkaline phosphatase (PCR product presequencing kit, Amersham). Sequences were obtained from PCR templates using BigDye (Perkin-Elmer, Norwalk, Connecticut) terminator cycle sequencing chemistry and an ABI 377 automated DNA sequencer. All sequences were completely double-stranded for verification using reactions primed from the PCR primers (28S D2/D3) or a combination of PCR primers and internal primers (D18/D19 28S rDNA and ITS). Two internal sequencing primers were used for complete sequencing of the ITS region, < no. 264 (3,185–3,203 5′-CGTTTTTCATCGA-TACGCG) and > no. 389 (3,231–3,250 5′-TGCAGACGCTTA-GAGTGGTG), although only primers no. 93 and no. 264 were required to double-strand ITS-1. One internal sequencing primer was used for the D18/D19 28S rDNA region that was double-stranded (5,054–5,649), < no. 545 (5,662–5,685 5′-CCTTACCTACATTATTCTATCGAC). Sequence fragments were assembled and edited using Sequencher v 3.0 (Gene Codes, Ann Arbor, Michigan). Invariant flanking regions, corresponding to the PCR primers, were removed from the sequences before sequence alignment and analysis. Sequences were aligned initially using CLUSTAL X 1.53b (Thompson et al., 1997) and the resulting output was adjusted manually to improve homology statements.

RESULTS

Hookworm measurements and morphometric analyses

Males: There was an overall effect of host location (Alaska vs. California) on measurements of hookworms from northern fur seals ($P < 0.0001$) except for width and buccal capsule depth, which were not different. Therefore, separate analyses were used when data on male hookworms from northern fur seals from Alaska and California were compared with those from California sea lions. There was an overall difference between hookworms in northern fur seals (Alaska) and California sea lions ($P < 0.0001$). All measurements were different ($P < 0.05$) except for esophageal length, which was not significantly different. When data from northern fur seals (California) were compared with data from California sea lions (Table II), there were differences in all measurements taken ($P < 0.0001$), including body width ($P < 0.01$).

Females: There were no significant differences between measurements of hookworms from northern fur seals from Alaska and California. Therefore, data from female hookworms of

TABLE I. Diagnostic ribosomal DNA sequence data from individual hookworms. Apomorphies of *Uncinaria stenocephala* are unlisted except for those shared with fur seals and sea lions.

Individual ID/UCDNC voucher no.	Sex	Taxon	Host, host ID no.	Location	Variable rDNA characters* 123456	D2/D3 accession no.	D18/D19 accession no.	ITS accession no.
1,818/3,689	F	*U. stenocephala*	Arctic Fox	St. Paul Island, Alaska	TGCCGC	AF217867	AF257712	AF194145
1,572/3,671	F	*Uncinaria*	Northern fur seal #5	San Miguel Island, California	CTCCGC	AF217880	AF257715	AF217892
1,571/3,670	M	*Uncinaria*	Northern fur seal #5	San Miguel Island, California	CTCCGC	AF217872	AF257714	AF217893
1,579/3,676	F	*Uncinaria*	Northern fur seal #6	San Miguel Island, California	CTCCGC	AF217869	AF257720	AF217894
1,580/3,677	M	*Uncinaria*	Northern fur seal #6	San Miguel Island, California	CTCCGC	AF217874	AF257721	AF217895
1,581/3,678	F	*Uncinaria*	Northern fur seal #6	San Miguel Island, California	CTCCGC	AF217886	AF257722	AF217896
1,582/3,679	F	*Uncinaria*	Northern fur seal #6	San Miguel Island, California	CTCCGC	AF217881	AF257723	AF217897
1,728/3,686	M	*Uncinaria*	Northern fur seal #15	San Miguel Island, California	CTCCGC	AF217871	AF257730	AF217890
1,730/3,688	F	*Uncinaria*	Northern fur seal #9	San Miguel Island, California	CTCCGC	AF217887	AF257731	AF217898
1,729/3,687	F	*Uncinaria*	Northern fur seal #12	San Miguel Island, California	CTCCGC	AF217878	—†	AF217899
1,577/3,674	F	*Uncinaria*	California sea lion #8	San Miguel Island, California	TA-AAT	AF217876	AF257718	AF217900
1,578/3,675	F	*Uncinaria*	California sea lion #8	San Miguel Island, California	TA-AAT	AF217882	AF257719	AF217901
1,576/3,673	F	*Uncinaria*	California sea lion #8	San Miguel Island, California	TA-AAT	AF217877	AF257717	AF217902
1,718/3,680	M	*Uncinaria*	California sea lion #24	San Miguel Island, California	TA-AAT	AF217875	AF257724	AF217889
1,575/3,672	M	*Uncinaria*	California sea lion #8	San Miguel Island, California	TA-AAT	AF217888	AF257716	AF217903
1,720/3,681	F	*Uncinaria*	California sea lion #43	San Miguel Island, California	TA-AAT	AF217883	AF257725	AF217904
1,724/3,683	F	*Uncinaria*	California sea lion #33	San Miguel Island, California	TA-AAT	AF217873	AF257727	AF217905
1,725/3,684	F	*Uncinaria*	California sea lion #32	San Miguel Island, California	TA-AAT	AF217885	AF257728	AF217906
1,726/3,685	M	*Uncinaria*	California sea lion #32	San Miguel Island, California	TA-AAT	AF217870	AF257729	AF217907
1,568/3,669	M	*Uncinaria*	California sea lion #43	San Miguel Island, California	TA-AAT	AF217879	AF257713	AF217908
1,722/3,682	M	*Uncinaria*	California sea lion #49	San Miguel Island, California	TA-AAT	AF217884	AF257726	AF217909
2,361/3,690	F	*Uncinaria*	California sea lion #1/4	San Nicolas Island, California	TA-AAT	—‡	AF257732	AF217910
2,362/3,691	F	*Uncinaria*	California sea lion #1/4	San Nicolas Island, California	TA-AAT	—‡	AF257733	AF217911
2,363/3,692	M	*Uncinaria*	California sea lion #2/4	San Nicolas Island, California	TA-AAT	—‡	AF257734	AF217912
2,364/3,693	F	*Uncinaria*	California sea lion #2/4	San Nicolas Island, California	TA-AAT	—‡	AF257735	AF217913
2,366/3,694	F	*Uncinaria*	California sea lion #2/4	San Nicolas Island, California	TA-AAT	—‡	AF257736	AF217914

[a] Variable characters are reported only for polymorphisms fixed within the populations sampled among sea lions and fur seals (1, D18/D19 position 267; 2, ITS-1 position 103; 3, ITS-1 position 122; 4, ITS-1 position 205; 5, ITS-1 position 380; 6, ITS-1 position 394).

† Sequence not determined because of repeated failure of PCR amplification.

‡ Sequence not determined because of the absence of diagnostic differences among pinniped hookworms acquired and sequenced previously (see text).

TABLE II. Measurements of 25 male *Uncinaria*, each from northern fur seal pups from Alaska and California, and from California sea lion pups, from California.

Hosts	Length (mm)	Width (μm)	Buccal capsule (μm)	Esophageal length (μm)	Spicules (μm)
Northern fur seals, Alaska					
Mean	7.21	300.20	185.25	824.72	468.62
Range	5.89–8.69	252.80–331.80	162.50–231.25	793.16–948.00	410.80–553.00
Standard deviation	0.55	19.35	13.19	44.88	35.68
Northern fur seals, California					
Mean	6.82	300.20	183.00	794.34	508.74
Range	5.61–8.10	237.00–347.60	156.25–206.25	742.60–805.80	426.60–616.20
Standard deviation	0.57	25.80	11.90	11.75	45.63
California sea lions, California					
Mean	8.33	319.16	203.50	809.64	686.35
Range	6.52–10.29	237.00–363.40	168.75–213.25	791.26–820.02	616.20–758.40
Standard deviation	0.77	24.56	15.42	7.65	40.30

northern fur seals from both locations were combined for comparisons with hookworms from California sea lions. There was an overall effect of host species, northern fur seal versus California sea lion ($P < 0.0001$). Total length, width, buccal capsule depth, and esophageal length were different ($P < 0.0001$), but distance of the vulva from the posterior end and tail length were not (Table III).

28S rDNA sequence data and analysis

Ribosomal sequences from individual *Uncinaria* specimens have been deposited in GenBank (accession numbers in Table I). The D2/D3 28S rDNA region for all hookworms sampled from fur seals and sea lions was 561 nucleotides (nt) long (4 bases adjacent to 3′-end of primer no. 501 could not be unambiguously determined in all individuals, and thus 557 nt were deposited and compared in all individuals). The same region from *U. stenocephala* was also 561 nt, whereas the D2/D3 rDNA of *N. americanus* (GenBank AF217868) was 570 nt. The D2/D3 rDNA sequences of 20 individual hookworms from California sea lions and northern fur seals (representing sympatric rookery sites) were invariant, with the exception of 2 worms (ID numbers 1,576 and 1,578, Table I) taken from the same host individual that were polymorphic (A/G) at position 79 in the multiple sequence alignment (alignment deposited in TreeBASE; Sanderson et al., 1994). Because of the invariant nature of these pinniped hookworm sequences, nematodes from sea lions on San Nicolas Island (the allopatric site) were not sampled for the D2/D3 rDNA region (Table I). The pinniped hookworms differed from *U. stenocephala* at 12 alignment positions (10 transitions, 2 transversions) and from *N. americanus* at 33 positions (24 transitions, 9 transversions) for the D2/D3 rDNA region. Average base composition for all taxa was 0.23 (A), 0.22 (C), 0.31 (G), and 0.24 (T). An among-taxon test revealed no significant departure from base frequency homogeneity (chi-square $P = 1.00$). Four insertion/deletion events including 2 3-base insertions, 1 2-base insertion, and 1 1-base

TABLE III. Measurements of 25 female *Uncinaria*, each from northern fur seal pups from Alaska and California, and from California sea lion pups, from California.

Hosts	Length (mm)	Width (μm)	Buccal capsule (μm)	Esophageal length (μm)	Vulva (mm)*	Tail (μm)†
Northern fur seals, Alaska						
Mean	11.70	375.38	221.99	815.07	4.59	233.75
Range	9.77–13.83	331.80–426.60	187.50–256.25	804.22–840.05	3.44–5.61	187.50–300.00
Standard deviation	1.03	24.25	17.03	7.23	0.53	23.45
Northern fur seals, California						
Mean	11.61	367.88	222.00	818.06	4.73	224.75
Range	9.48–13.02	300.20–442.40	168.73–250.00	805.80–832.66	4.02–5.93	156.25–268.75
Standard deviation	0.88	33.12	23.05	6.32	0.45	19.89
California sea lions, California						
Mean	12.61	412.70	241.50	828.85	4.55	214.75
Range	11.06–13.79	347.60–537.20	218.75–268.75	821.60–837.40	3.48–5.53	162.50–281.25
Standard deviation	0.82	41.13	12.74	4.32	0.46	32.62

* Distance from posterior end.
† Length.

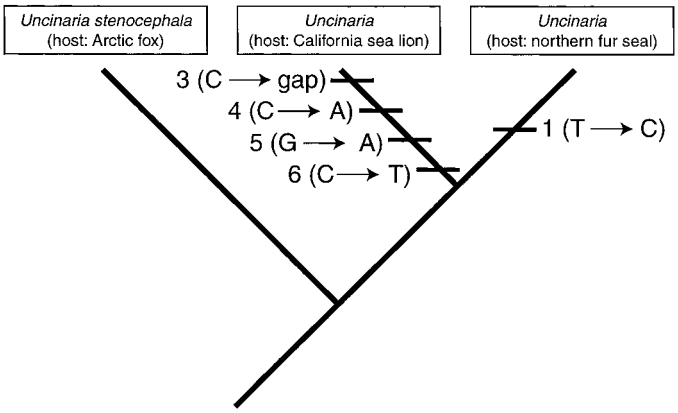

FIGURE 1. Evidence of character evolution and lineage independence among *Uncinaria* parasitizing Arctic foxes (*Alopex lagopus*), California sea lions (*Zalophus californianus*), and northern fur seals (*Callorhinus ursinus*) on the basis of outgroup comparison and parsimony analysis. Of 6 polymorphic rDNA nucleotides fixed within sampled populations (Table I), 1 D18/D19 character is autapomorphic for *Uncinaria* from northern fur seals (character 1, a transition). Four ITS-1 rDNA sites (characters 3–6) are autapomorphic for *Uncinaria* from California sea lions (character-state changes include 2 transitions, 1 transversion, and 1 deletion). Character evolution for 1 potentially informative ITS-1 site (character 2, Table I) is ambiguous by parsimony criteria. The exclusive fixation of unique, derived character-states provides evidence of historical, and thus prospective, lineage independence for species delimitation.

insertion account for length variation between *N. americanus* and the other taxa. The region that was double-stranded for the D18/D19 28S rDNA sequences was 596 nt in all sampled hookworms. The D18/D19 sequences of all individual hookworms from California sea lions and northern fur seals showed 1 difference (position 267, a transition) that was partitioned without exception according to host (Table I). Cladistic analysis of position 267 using outgroup comparison (*U. stenocephala* outgroup) provided evidence for the fixation of a derived character state among individual hookworms from northern fur seal hosts (Fig. 1). Two additional sequence differences (transitions between C and T) were observed between the pinniped hookworms and *U. stenocephala.*

ITS rDNA sequence data and analysis

The amplified region that included the ITS rDNA subunits (partial 3′-end 18S, ITS-1, 5.8S subunit, ITS-2 and partial 5′-end 28S) from northern fur seals was 836 nt long (6 nt adjacent to the 5′-end 18S primer and 8 nt adjacent to 3′-end 28S primer could not be unambiguously determined, thus 822 nt were deposited and compared among taxa). In California sea lion hookworms and *U. stenocephala,* the amplified region was 835 nt (821 nt deposited and compared). However, the same region in *N. americanus* (GenBank AF217891) was 1,090 nt (1,076 deposited and compared). Among *Uncinaria,* the ITS-1 region was 365 nt (taxa from fur seals and *U. stenocephala*) or 364 nt (taxa from sea lions); ITS-2 was 225 nt in the pinniped *Uncinaria,* and 224 nt in *U. stenocephala.* The multiple sequence alignment for pinniped *Uncinaria* and outgroup taxa (deposited in TreeBASE) contained 26 indels involving 1 nt position, and 32 indels of more than 1 nt position. These numerous, large indels resulted in several regions of ambiguity in the multiple alignment. Because of doubts about positional homology statements for the *N. americanus* sequence relative to other taxa, this outgroup was excluded from additional comparisons; this reduced the number of alignment indels to 4, all of which involved single nucleotide events. Seven ITS sites were found to vary among hookworms sampled from fur seals and sea lions. Five of the 7 variable sites were in the ITS-1 region, and these 5 dimorphic characters were partitioned without exception according to host (Table I). Differences between these host-specific sequences involved 2 transitions, 2 transversions, and an insertion/deletion event. Positions and differences for these sites (character for fur seal hookworms/sea lion hookworms) were: 103 (T/A), 122 (C/gap), 205 (C/A), 380 (G/A), and 394 (C/T), with positions numbered according to the multiple alignment of *Uncinaria* taxa (without *N. americanus*). All 4 nt substitutions involved either an adenine or thymine, and overall, ITS-1 was A-T rich (A = 0.21; C = 0.22; G = 0.27; T = 0.30) among *Uncinaria,* and among *Uncinaria* plus *N. americanus* (A = 0.23, C = 0.21, G = 0.25, T = 0.31). An among-taxon comparison revealed no significant departure from base frequency homogeneity (chi-square for *Uncinaria* only, $P = 0.95$; for *Uncinaria* plus *N. americanus* $P = 0.89$). Two ITS-2 sites were found to vary among hookworms sampled from northern fur seals and California sea lions. These sites included 2 putative autapomorphies (one for hookworms from northern fur seals, the other for hookworms from California sea lions); however, additional hookworms were not sequenced for ITS-2 because sufficient informative character data were obtained from other rDNA regions.

Cladistic analysis of the 5 variable ITS-1 characters using outgroup comparison (*U. stenocephala* outgroup) revealed that 4 of the sites (positions 122, 205, 380, and 394) provided evidence for the fixation of a derived character state in hookworms from California sea lion hosts (Fig. 1).

DISCUSSION

The ITS rDNA region appears to be a useful molecular marker for investigating evolutionary patterns among nematode taxa at various levels of the taxonomic hierarchy (Powers et al., 1997). Hookworm species from different genera, and populations assumed to represent single species, have been investigated by several authors using ITS sequences, or specific tests developed from ITS PCR products (Gasser et al., 1996, 1998; Monti et al., 1998; Romstad et al., 1998). Other molecular markers, including the 3′ untranslated region of a cAMP-dependent protein kinase (Hawdon, 1996) and the cysteine protease AcCP1 (Mieszczanek and Wedrychowicz, 1999), have been used to discriminate genera and species of hookworms. The D2/D3 expansion domains of the nuclear 28S rDNA subunit are another sequence region that has been successfully used for diagnosing nematode species, including specimens that have been briefly fixed using formalin (Thomas et al., 1997).

The present study permits a direct comparison of the 28S and ITS regions of the nuclear rDNA repeat among some Ancylostomatidae. The pattern of substitution in these regions is clearly different, with the ITS region evolving at a higher rate, which is consistent with expectations of lower constraint for spacer sequences relative to sequences of the functional ribosome such as the D2/D3 region (Baldwin et al., 1995). The ITS region also

appears less constrained with respect to substitution type. For example, on the basis of observed (uncorrected) substitutions, the D2/D3 rDNA region revealed the expected transition(ti): transversion(tv) bias (ti:tv of 2.11 [*U. stenocephala* vs. *N. americanus*] to 5.0 [*U. stenocephala* vs. hookworms of California sea lions and northern fur seals]), whereas the ITS region showed less transition bias (ti:tv of 1.0 [hookworms of California sea lions vs. hookworms of northern fur seals] to 2.33 [hookworms of California sea lions vs. *U. stenocephala*]). In addition, the alignment of the D2/D3 region contained only 4 indels (2 3-base, 1 2-base, and 1 1-base) relative to 36 substitution events. By contrast, in the alignment of the ITS region including *N. americanus*, there were 58 indels relative to 112 substitution events. This comparison of indels to substitutions assumes that multiple bases comprising an indel represent a single insertion–deletion event. If the multiple-base indels result from the cumulative total of separate single events, the proportion of indels to detectable single base substitutions would be even greater for the ITS region (maximum of 271 indel events to 112 single nucleotide substitutions). These findings suggest that ITS rDNA is less constrained relative to type and rate of substitutions than the D2/D3 rDNA among these hookworms.

Adult *Uncinaria* from northern fur seal pups are significantly different from those in California sea lion pups on the basis of morphometry; this difference was found irrespective of which locality was used for comparison of the northern fur seal hookworms (California or Alaska). Although Baylis (1933, 1947) described certain qualitative differences between *U. lucasi and U. hamiltoni*, we focused our morphological study on quantitative features, because the structures discussed by Baylis varied by angle of view, and seemed to require subjective interpretation. Our sampling revealed no sequence differences for ITS-1 between allopatric populations of adult hookworms parasitizing California sea lions. Sequences from hookworms representing allopatric populations from northern fur seals were not determined (Alaska specimens collected in 1960 and used for morphometric analysis could not be amplified successfully by PCR, presumably because of DNA damage resulting from fixation and storage effects); however, genetic comparisons of hookworm samples from California and Alaska are warranted because male specimens from these hosts showed significant differences for certain measurements. Genetic and morphometric variation among individual hookworms sampled from sympatric northern fur seals and California sea lions were partitioned exclusively according to host species. For example, the 6 rDNA differences (Table I) among individual hookworms taken from sympatric northern fur seals and California sea lions are fixed within each respective host, but vary between host species. Thus, these genetic data provide independent evidence that these 2 diagnosable hookworm taxa do not develop to adults in the intestines of both California sea lions and northern fur seals; instead, each taxon appears restricted to a single host species. Likewise, these 2 taxa are diagnosable on the basis of morphometric data, providing additional evidence for host specificity.

In addition to being diagnosably different (and thus satisfying the criterion of phylogenetic species sensu Nixon and Wheeler, 1990; Wheeler, 1999), there is evidence that these 2 *Uncinaria* lineages from otariids have been evolving independently in the past and, therefore, can be delimited as species using more theoretically rigorous criteria. Regarding the status of species, we make the distinction between diagnosis and delimitation as the latter having meaning in an explicit evolutionary context. Wary of confusing what species are with how they can be discovered, we advocate searching for ontologically ''real'' species (our preference being the evolutionary species concept of Wiley and Mayden, 2000) by using amended discovery operations of the phylogenetic species concept (Frost and Kluge, 1994; Adams, 1998). This approach focuses on ''how do we know we have species?'' (epistemology) within the context of the theoretical or ontological framework provided by our species concept, i.e., ''do we have species?'' The former (epistemological) question is typically addressed without an explicit theoretical framework when comparisons of pairwise sequence differences (or phenetic similarity) are used to assess if a certain taxon merits species status (e.g., Hung et al., 1997, 1999; Romstad et al., 1998; Gasser et al., 1999; Heise et al., 1999). For example, in a sequence-based study of equine strongylids (*Cylicocyclus*), Hung et al. (1997) stated that they ''compared DNA sequences . . . of rDNA of *C. ashworthi* and *C. nassatus* in order to provide molecular data that these taxa represent different species, and to establish a rapid polymerase chain reaction (PCR)-based method for their delineation.'' This near-tautological activity of searching for data that support or ''delineate'' the a priori expectation of species does not involve potential refutation, but verification, and is of limited scientific merit (Popper, 1968; Lakatos, 1970; Kluge, 1997). No doubt the impetus for some recent studies of parasites has been to provide molecular markers that discriminate among morphologically indistinguishable life history stages, e.g., eggs or juveniles, for species that are otherwise accepted as ''well established'' on the basis of distinctiveness of adults and taxonomic differential diagnoses (Gasser et al., 1998; Monti et al., 1998; Mieszczanek and Wedrychowicz, 1999). However, even this approach to using molecular data for verification of ''species'' has potential caveats, e.g., if the differential diagnosis of the original ''well-established'' species was confounded by host-induced morphological differences or other pitfalls.

Studies that seek to identify and use all observed sequence differences to define species (e.g., Hung et al., 1997), typically through application of a genetic ''yardstick'', are problematic for several additional reasons. Potential problems include variation in the rate of sequence evolution among lineages, which may confound interpretations of pairwise sequence divergence, and the requirement to set a boundary defining the minimum amount of sequence divergence that merits species status. For example, *Uncinaria* taxa from pinnipeds display simple sequence differences (formula of Newton et al., 1998) of 1.1% for ITS-1 and 0.88% for ITS-2, whereas the average difference between these pinniped hookworms and *U. stenocephala* is 7.4% for ITS-1 and 10.6% for ITS-2. How can such measures of genetic differentiation be interpreted with respect to evaluating species status? Even if the taxa under consideration have a constant rate of sequence evolution for the gene investigated, interpretation of sequence divergence for exemplar taxa may be confounded by the taxa sampled. Is our ''genetic yardstick'' comparison based on pairs of sister species, or is information available only for more distantly related congeners? Comparison of pairwise or average similarity between taxa also ignores the risks of defining evolutionary groups (in this case species)

on the basis of all types of differences and similarities, including plesiomorphies. Perhaps, most importantly, using a genetic yardstick for defining species entails the assumption that some characteristic (minimum) threshold of genetic change is a requisite property of species; however, this assumption is not consistent with empirical evidence on the genetics of species differences (Templeton, 1981). The lack of consistent correlation between genetic differentiation and taxonomic rank indicates that interpreting the genetics of species differences requires a different emphasis, one that focuses on identifying evolutionary taxa and their novelties (Cracraft, 1989).

We view species as time-extended individuals (lineages) (Ghiselin, 1974, 1987, 1997; Hull, 1978; but see also Baum, 1998) and require evidence of historical lineage independence (in the form of autapomorphic characters) to delimit species. Testing the hypothesis of lineage independence in any particular case requires phylogenetic interpretation of data and the potential for failure to recover such lineages (Adams, 1998). This method does not assume that only lineages with autapomorphies are species (which would conflate an epistemological approach with a theoretical concept of what species are); rather, this approach represents an objective method for using character-state data (such as nucleotide sequences) to delimit species by hypothesis testing (see Adams, 1998). For example, assuming *U. stenocephala* shares a most recent common ancestor with the 2 diagnosable hookworms parasitizing otariids, the different homologous character states shared by these taxa can be polarized. Of 5 ITS-1 sites with fixed differences between the hookworms sampled from the 2 species of otariids, 4 have shared states between *U. stenocephala* and *Uncinaria* from northern fur seals. The most parsimonious interpretation of these ITS-1 characters is that hookworms from California sea lions have evolved 4 unique, derived character states (autapomorphies). However, these 4 autapomorphic characters are not sufficient to distinguish hookworms from California sea lions and northern fur seals as unique in an evolutionary sense (although the ITS-1 sites provide diagnosable differences between hookworms from each host). The necessary additional data comes from cladistic analysis of the D18/D19 site, which provides evidence of an autapomorphy for hookworms from northern fur seals (Fig. 1). Thus, hookworms from California sea lions and northern fur seals each have one or more autapomorphies as evidenced from cladistic analysis of these rDNA data. The fact that these autapomorphic states are fixed among sampled individuals, and not shared with any other lineage, is evidence of historical lineage exclusivity. Such historical independence supports the hypothesis that these lineages represent distinct species. Thus, in contrast to a single, widespread hookworm species with multiple host species, these data support the notion of 2 distinct hookworm species as proposed in the early descriptions of Baylis (1933, 1947). One potential caveat to this interpretation is that although autapomorphies have been inferred for the 2 otariid hookworm species in question, these unique derived states cannot be unquestionably determined until their homologues are examined among all other species of *Uncinaria*. Likewise, confidence in the exclusive and fixed nature of these autapomorphies would be increased by sampling hookworms and hosts from a larger geographic range, although the geographic range for parasite transmission (rookeries) is much more restricted than host ranges in the ocean.

In the context of this evolutionary interpretation, it is also notable that California sea lion pups that died at rookeries on San Miguel Island had twice the number of hookworms in comparison with northern fur seal pups, and there appeared to be differences in hookworm location, pathogenesis (Lyons et al., 1997), and longevity (Lyons et al., in press) between these hosts. It was previously unclear if this was due to differential effects of 1 hookworm species in 2 host species, or as a consequence of distinct species of hookworms in these 2 hosts. We provide evidence that *Uncinaria* of northern fur seals and California sea lions are 2 distinct species. However, we cannot confirm with confidence that the species in northern fur seals and California sea lions correspond to *U. lucasi* and *U. hamiltoni*, respectively. Although the description of *U. lucasi* is consistent with specimens obtained from hosts collected in California, analysis of morphometric data revealed significant differences between certain characters of male hookworms obtained from northern fur seals collected in California and Alaska. Because we lack sequence data for the Alaska specimens, we cannot use genetic data to assess topotype specimens of *U. lucasi*. Likewise, the description of *U. hamiltoni* is based on specimens obtained from a South American sea lion, and morphological observations suggest that hookworms of California sea lions may be intermediate between those found in northern fur seals and South American sea lions (Baylis, 1947; Dailey and Hill, 1970). Baylis (1947) concluded, ''. . . it does not seem desirable to regard the Californian species as distinct until it has been more fully investigated (p. 311).'' Having done so, the onus of evidence must be directed to comparative analysis of other hookworms infecting pinnipeds.

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